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Applicant:	Jessamine M.K. Lee, et al.
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DECLARATION OF PRIOR INVENTION IN THE UNITED STATES TO OVERCOME CITED PATENT OR PUBLICATION UNDER 37 C.F.R. §1.131

Sir:

I, George M. Whitesides, declare that:

1. This Declaration is to establish completion of the invention as recited in at least claims 1-7, 9-13, 15-36, 53-65, 77, and 85.

2. I am an inventor of the above-identified application and of the claims specifically mentioned in Paragraph 1. I am a Professor of Chemistry and Chemical Biology at Harvard University (the assignee of this application), and I served as the lead investigator in research leading to the invention(s) described and claimed in this application. As an inventor and Harvard employee,

I derive monetary benefit from licensing and/or other revenues associated with this patent application and/or any patent issuing therefrom.

3. I, and co-inventors, conceived and reduced to practice the invention as defined in at least claims 1-7, 9-13, 15-36, 53-65, 77, and 85 prior to April 28, 2001, the date of publication of Gao et al., "Integrated Microfluidic System Enabling Protein Digestion, Peptide Separation, and Protein Identification", *Anal. Chem.*, 73, 2648-2655 (2001) which was cited in the Office Action mailed January 22, 2009, by evidence of the attached manuscript shown in the Appendix. Prior to April 28, 2001, I received this manuscript from my co-inventors. The date on the manuscript has been redacted in the Appendix. The manuscript was eventually submitted with revisions to the journal *Analytical Chemistry* and published as Ismagilov et al., "Microfluidic Arrays of Fluid-Fluid Diffusional Contacts and as Detection Elements and Combinatorial Tools", *Anal. Chem.* 73, 5207-5213 (2001).

5. All statements made herein of my own knowledge are true, and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and like made in a declaration are punishable by fine or imprisonment or both under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Full name of undersigned inventor: George M. Whitesides

Signature: _____

Date: 3/27/09

Citizenship: U.S.

Residence: 124 Grasmere Street, Newton, MA 02458

Appendix

[redacted]

Dear George,

[redacted]

Sincerely,

[redacted]

[redacted]

Microfluidic Arrays of Fluid-Fluid Diffusional Contacts as Detection Elements and Combinatorial Tools

Rustem F. Ismagilov[+], Jessamine M.K. Ng[+], Paul J. A. Kenis, and

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[+] These authors have contributed equally to this work.

Suggested Journal: Anal. Chem.

Abstract:

This paper describes microfluidic systems with which to investigate multiple chemical or biochemical interactions in a parallel format. These three-dimensional systems are fabricated in two different layers by crossing two sets of microfluidic channels at right angles. Solutions of the reagents are placed in the channels, and in different modes of operation, these solutions can be either flowing or stationary--the latter is especially important when viscous gels with immobilized reagents are used. At every crossing the channels are separated by a single membrane, or a composit separator comprising a membrane, a microwell, and a second membrane. These components allow diffusive mass transport and minimize convective transport through the crossing. Each crossing serves as an element where chemical or biochemical interactions take place; interactions can be detected by monitoring changes in fluorescence and absorbance. These all-organic systems are straightforward to fabricate and to operate, and may find applications as portable microanalytical systems and as tools in combinatorial research.

[redacted]

Introduction

This paper describes simple, three-dimensional microfluidic networks designed to investigate interactions of two sets of reagents in parallel using an array format. These networks provide a new type of system in which to carry out arrayed analysis and combinatorial processes.

Each set of solutions of reagents is placed in a set of parallel channels. The two sets of channels are oriented at 90° to each other, separated by a thin membrane (or, in some experiments, a more complicated structure) in such a way that the species dissolved in the fluids of two crossing channels can diffuse from one channel to another (Figure 1). In this paper we describe two types of systems. In the first--the "membrane system"--only a polycarbonate membrane separates crossing channels (Figure 1A). In the second--the "microwell system"--the channels are separated by composit structure consisting of a a membrane, a microwell, and another membrane (Figure 1B). At every crossing of the channels, the pores of the membrane of the first system and the microwells of the second system provide small volumes of stationary fluid into which reagents can diffuse from the two channels, and where chemical interactions can take place, but through which little or no *convective* fluid exchange occurs. This absence of convective flow through the crossing is essential, because it allows localization of the products of the reaction between the two reagents, and prevents cross-contamination of reagents in different channels. Convective flow through crossings can be minimized in two ways: first, by minimizing pressure gradients across the crossing, and second, by building the crossing to have a high resistance to fluid flow; we use both of these approaches.[1]

We demonstrate three modes of operation of these systems: i) one in which the fluids in both channels are flowing; ii) a second in which fluids in one set of channels are immobilized (as gels); iii) a third in which fluids are stationary in both sets of channels. To pump the fluids through the channels, we used pressure-driven flow generated either with a syringe pump or by controlling the height of the fluid column in the inlets and relying on gravity. Changes in fluorescence, absorbance, and precipitation reveal interactions between the species in the crossing channels. We believe that detection schemes based on changes in refractive index and in light scattering in the regions where channels cross will also provide methods of detecting these interactions.

A number of two-dimensional networks have been described for bringing fluids into contact inside microchannels without convective mixing. Yager has illustrated a system--the "T sensor"--in which species present in two adjacent streams flowing laminarly, in parallel, react by diffusion across the fluid-fluid interface.[2,3] Previously we also have described chemical reactions at interfaces of pairs of laminar streams.[4-6] In such two-dimensional systems, every stream can be brought into contact with a maximum of two other streams (one on each side). The systems described in this paper are three-dimensional, and allow a large number of fluid-fluid contacts in an easily fabricated microfluidic array.

These microfluidic networks are fabricated from polydimethylsiloxane (PDMS) using rapid prototyping, a technique that is well suitable for the fabrication of three-dimensional microstructures.[7] PDMS is an attractive material for microfluidic devices intended for biological analysis because it is optically transparent, flexible, and impermeable to liquid water, and because it can be easily integrated with organic

polymers.[8] We were therefore easily able to incorporate into the microfluidic structures an important component--an organic membrane that separates the flowing streams.[9] This sort of membrane provides multiple functionalities: it reduces convective transport of one fluid stream into the other; it controls the distance over which species must diffuse in the area of contact; it provides a capability for selection for or against species present in the microchannel (e.g. by molecular weight, size, charge, partition coefficient, or bioaffinity).

Experimental

Reagents. Nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, 5-bromo-4-chloro-3-indolyl galactoside, fluo-3, ELF-97 phosphate, ELF-97 acetate, and ELF-97 β-D-glucuronide were purchased from Molecular Probes and used as received. Enzymes β-galactosidase (from *Escherichia coli*, 250-600 units per mg), alkaline phosphatase (from bovine intestinal mucosa, 23 units per mg), esterase (from porcine liver, 150 units per mg), and β-D-glucuronidase (from *Escherichia coli*, 1000-5000 units per mg) were purchased from Sigma-Aldrich.

Staphylococcus aureas (provided by Jean Lee, Channing Laboratory at the Harvard Medical School) were grown in BBL™ Trypticase™ Soy Broth (Becton Dickinson and Company) by incubation at ~37°C for ~24 hours. Bacteria were washed and resuspended in PBS buffer (Sigma-Aldrich). Staphylosłide™ Latex beads were purchased from VWR.

Substrates for the enzymes were immobilized in agarose gel by heating a 2.5% agarose solution in water to ~70°C and then mixing it with an equal volume of the solution of the substrate. While still hot, 5 μL of the resulting mixture was injected into

the channels and the device was cooled to 4°C for at least 5 minutes in order to solidify the gel.

Fabrication. The key elements of the two types of networks are that the channels cross in multiple planes, but are separated by a membrane (Figure 1A), or a more complex structure (for a example, membrane, a microwell, and another membrane; Figure 1B). The former system can be fabricated with single steps of alignment and sealing, and uses two PDMS molds bearing the channel structures, separated by a single 10-μm-thick polycarbonate membrane having ~ 0.1-μm vertical pores. The latter system requires two steps of alignment and sealing. In this system, the PDMS molds are separated by two polycarbonate membranes that sandwich a thin PDMS membrane bearing microwells.

To allow introduction of fluid samples into the channels, in both systems the access holes were punched through PDMS with a 21-gauge syringe needle that had been cut in order to remove its slanted tip, polished, and then sharpened. Fluids were supplied either through polyethylene tubing inserted into the access holes, or by placing drops of fluid directly into the access holes. Although we filled the channels by hand, they are easily compatible with inkjet printing or robotic dispensing.

We found that in our devices, a small gap was formed at the edge of the membrane between the two PDMS molds (Figure 2A). This gap might, in principle, provide a path for diffusive or convective exchange of fluids or solutes between parallel channels, and therefore cause cross-contamination of the reagents in these channels. Although the presence of this gap did not affect the experiments described in this paper, we developed a simple method with which this gap could be blocked. In this method, the edges of the membrane used in the fabrication of the device were wetted with liquid

PDMS prepolymer, the device was sealed and placed in the oven at 65 °C for 1 hour to cure PDMS inside the gap. Devices fabricated according to this procedure did not show any leakage along the edge of the membrane (Figure 2B).

Results and Discussion

Addressability of individual crossings. To demonstrate that the individual crossings can be addressed independently, we detected calcium(II) ions in a channel of one layer of a 5x5 array using a fluorescent probe, fluo-3, in a channel in the other layer; the other eight channels were filled with water (Figure 3). In both systems, fluorescence was observed only at the crossing of the channels containing calcium(II) ions and fluo-3. Figure 3A shows that in the single membrane system there is weak flow through the crossing that leads to leakage of fluorescent calcium(II)/fluo-3 complex into the channel. This problem is not observed in the microwell system (Figure 3B).

Both systems were designed to balance fluid pressure, and thus to prevent convective fluid flow, across each crossing of the channels (Figure 3). The distances from any crossing to the inlet is equal in the two layers of channels, and so is the distance from the crossing to the outlets. Assuming that channels are uniform in dimensions and that the pressure is equal at all inlets and at all outlets, this design ensures that there is no pressure differential across the crossings. Variations in the pressures, and imperfections in the dimensions of the channels, are sufficient to generate observable flow in the membrane system.

In the membrane system, the chemical reactions take place in small pores (~ 0.1 μm in diameter) of a 10-μm-think membrane; in the microwell system the reactions take place in 200-μm-thick microwells. The advantage of the former system is rapid equilibration by

mass transport, since diffusion must transport molecules over only small distances. The advantage of the latter is larger reaction volume and longer optical path, and higher observed intensity of fluorescence.

Agglutination. We used the microwell system to demonstrate detection of bacteria by agglutination of test beads (Figure 4A). Two polycarbonate membranes with ~ 1.0-μm pores separated the microwell from the upper channels, containing Staphyloslide™ Latex beads, and the bottom channel, containing *Staphylococcus aureas*. The membranes were permeable to both beads[10] and bacteria.[11] The upper channels were filled with two types of blue Staphyloslide™ Latex beads. The first channel was filled with a suspension of test beads--those coated with human fibrinogen and immunoglobulin G (IgG) on their surfaces. The second channel was filled with a suspension of control beads--those without fibrinogen or IgG on their surfaces. The bottom channel was filled with a suspension of *Staphylococcus aureas* (ca. 10^9 bacteria/mL).[12] The pressure was made slightly higher in the channel with bacteria relative to the channels with beads to maintain weak flow of bacteria into the channels containing the beads. The fluid flow was maintained by gravity, and the pressure in the channels was regulated by controlling the height of fluid in the inlet reservoirs. When bacteria come into contact with the test beads, protein A on the surface of the bacterial cell wall binds to the Fc region of IgG on multiple beads, and the beads agglutinate.[13] Agglutination was visible only at the crossing where the test solution and the bacteria came into contact (Figure 4B). Agglutination occurred on the surface of the membrane in the channel containing the beads.

Incorporation of gels into the membrane system. To minimize problems with cross-flow in the membrane system, we embedded one set of reagents in an agarose gel (Figure 5A). Since the gel does not flow under pressure, and since the hydrodynamic resistance to flow across the gel is high, there is no danger of cross-flow between channels and pressure balancing becomes unimportant.

To demonstrate the use of a system that incorporated a functional gel,[14] we used a combination of colorimetric and fluorometric assays in a 5x5 channel array. The substrates were first immobilized in gels in one set of parallel channels, then the solutions of enzymes were injected into the other set of channels. In this experiment, the enzymes diffuse from their respective channels, through the membrane, and into the channels that contain gels with the substrates. Chromogenic substrates 5-bromo-4-chloro-3-indolyl galactoside (X-Gal)[15] and a combination nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP)[16] were used to detect the activities of β-galactosidase (β-gal) and alkaline phosphatase (AP)[17], respectively. Cleavage of these substrates generates a dark blue precipitate localized at the crossing of the channels.

Fluorogenic ELF-97[18] phosphate and acetate were used to detect enzymatic activity of alkaline phosphatase (AP) and esterase. When an enzyme cleaves the O-R bond of the ELF-97 substrate, an intensely fluorescent precipitate of ELF-97 alcohol forms at the crossing of the channels. This assay is especially suited for parallel screening because all substrates generate the same fluorescent compound upon cleavage. Figure 5B shows detection of esterase, β-galactosidase, and alkaline phosphatase (the latter by two independent assays).

In these systems, several samples can be analyzed simultaneously and in a small volume, and the results of the analyses can be compared directly. Each sample analyzed in this system leaves a pattern of signals that corresponds to its enzymatic activity. We believe that this method will be useful for analysis and identification of complex mixtures of enzymes in biological samples, and we propose to use it as a tool in biomedical assays.

Conclusion

All-organic microfluidic systems described in this paper--crossing channels with sandwiched functional components such as membranes and gels--provide convenient access to a range of chemical and biochemical assays in array format. In the mode of operation with fluids flowing in both sets of channels, these assays can be used for continuous monitoring. These new systems rely on both attractive features of rapid prototyping in PDMS--ease of fabrication of three-dimensional microstructures, and ease of incorporation of organic functional components such as membranes.

Assays in array format can be also conducted on a titer plate,[19] and using surface-bound reagents, as in DNA[20] and protein[21] arrays, although continuous monitoring is not possible in titer-plate assays. In the microfluidic arrays described in this paper, the number of wells required to be filled increases linearly with the size of the array; in the titer plate format, the number of wells increases quadratically. These systems do not require attachment of reagents to surfaces, in contrast to DNA arrays, although some systems require immobilization of reagents in gels. The main disadvantages of these microfluidic arrays are: i) they are more complex to fabricate than a titer plate; and ii) in operation, they require pressure balancing to control flow across the membrane.

We believe that these systems will both find practical applications and serve as research tools. They are inexpensive to fabricate, not fragile, and in the simplest version--using reagents for colorimetric detection immobilized in a gel--do not require any additional equipment or power source for visualization or pumping. These properties may make them especially attractive as portable microanalysis systems. Fabrication of these systems is straightforward and does not require highly specialized facilities; their design is flexible and can be easily modified. These additional properties could make them attractive as research tools in areas where parallel interactions among large number of components are studied, (e.g., in proteomics and diagnostics).

Acknowledgements

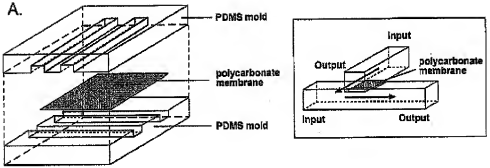
This work was supported by DARPA, and NSF ECS-0004030. We thank Steve Metallo (Harvard University) for helpful discussions.

[redacted]

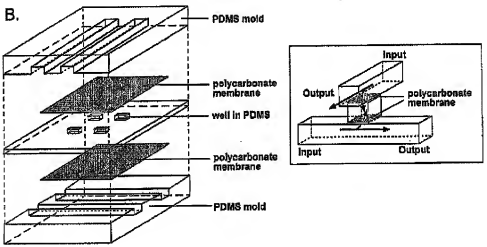
Figure 1

Schematic drawings illustrating fabrication of microfluidic systems for parallel screening. A. In the membrane system, crossing channels are separated by a single polycarbonate membrane. B. In the microwell system, crossing channels are separated by two polycarbonate membranes and a microwell. The membranes, which are 10-μm-thick with vertical pores ~ 0.1 μm in diameter, provide high resistance to convective flow through the crossing, but allow diffusion of reactants. C. A photograph of a typical device.

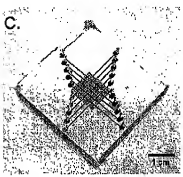
A.


PDMS mold
polycarbonate membrane
PDMS mold
Input
Output
polycarbonate membrane
Input
Output

B.


PDMS mold
polycarbonate membrane
well in PDMS
polycarbonate membrane
PDMS mold
Input
Output
polycarbonate membrane
Input
Output

C.



[redacted]

Figure 2

Fluorescent microphotographs of the membrane system at the crossing of a channel with the edge of the membrane. The channel was filled with 1 mM solution of fluorescein. A. In the system fabricated according to the procedure outlined in Figure 1A, there was a slight leakage of fluorescein along the edge of the membrane. B. This leakage could be prevented in the system where the edges of the membrane were treated with PDMS prepolymer prior to the assembly and sealing of the system. Upon curing, PDMS sealed the gap at the edge of the membrane and stopped the leakage. Both brightness and contrast of the original images were increased in order to make visible the leakage along the edge of the membrane shown in A. Under these conditions, fluorescent light scattered by the pores of the membrane outside of the channel becomes visible.

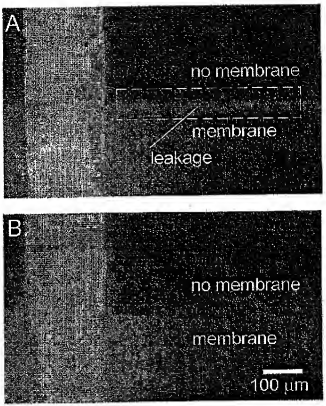
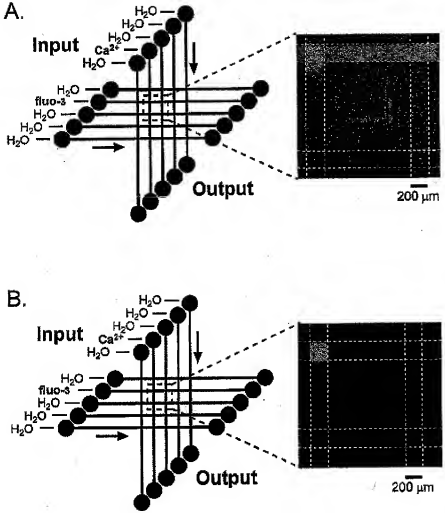

A
no membrane
membrane
leakage
B
no membrane
membrane
100 µm

Figure 3

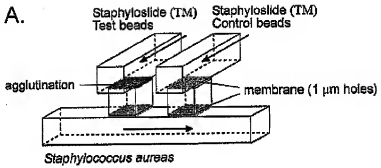
Fluorescent microphotographs illustrating addressability of individual microwells in a 5x5 array, fabricated as shown in Figure 1. Fluorescence is localized at the crossing of channels containing flowing aqueous solutions of $CaCl_2$ (1 mM) and fluo-3 (50 μM); no fluorescence is observed at the other 24 crossings (three are shown). In both experiments fluid flow was controlled with a syringe pump. A. Imbalances of pressure in the membrane system cause some $CaCl_2$ to be injected into the fluo-3-containing channel. Background fluorescence of fluo-3 is also visible. B. The microwell system is less sensitive to pressure imbalances than that based on membranes and gives higher fluorescence intensity and sensitivity due to the greater thickness of the region in which reaction occurs. The intensity of fluorescence from the microwell system (B) was normalized to the intensity of fluorescence from the membrane system (A) in order to facilitate comparison between the two.


A.
Input
H_2O
Ca^{2+}
fluo-3
Output
200 µm
B.
Input
H_2O
Ca^{2+}
fluo-3
Output
200 µm

[redacted]

Figure 4

Detection of *Staphylococcus aureas* by agglutination of Staphyloslide™ Test Latex beads with IgG immobilized on their surfaces. A. Schematic of agglutination in microfluidic channels. B. A microphotograph of the results of the experiment schematically shown in (A). Only agglutination of test beads, but not the control beads, was observed.


A.
Staphyloslide (TM)
Test beads
Staphyloslide (TM)
Control beads
agglutination
membrane (1 µm holes)
Staphylococcus aureas

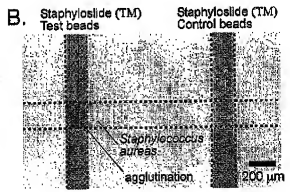
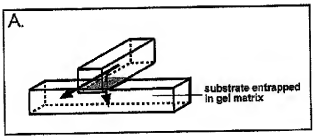
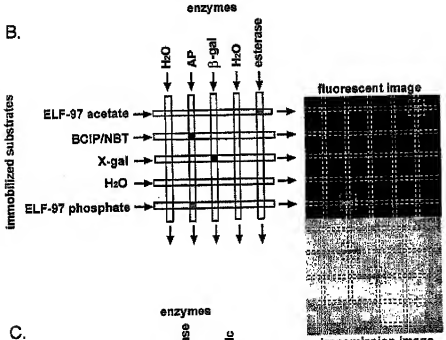
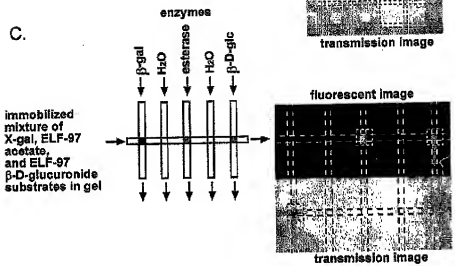

B.
Staphyloslide (TM)
Test beads
Staphyloslide (TM)
Control beads
Staphylococcus
aureas
agglutination
200 µm

Figure 5

Incorporation of functional components into the arrays of microwells. A. Schematic drawing of a 1x1 array of microfluidic channels of the membrane system showing a substrate entrapped in a gel matrix in one of the channels for B and C. B and C. Detection of enzymatic activity using fluorometric (top) and colorimetric (bottom) methods; fluid flow in the top set of channels was generated by gravity. B. A 5x5 array with substrates for enzymes immobilized in a 1.25% agarose gel. C. An immobilized mixture of X-gal, ELF-97 acetate, and ELF-97 β-D-glucuronide substrates in a 1.25% agarose gel used for parallel detection of enzymes.


A.
substrate entrapped
in gel matrix


B.
enzymes
H2O
AP
β-gal
H2O
esterase
immobilized substrates
ELF-97 acetate
BCIP/NBT
X-gal
H2O
ELF-97 phosphate
fluorescent image
transmission image


C.
enzymes
β-gal
H2O
esterase
H2O
β-D-glc
immobilized
mixture of
X-gal, ELF-97
acetate,
and ELF-97
β-D-glucuronide
substrates in gel
fluorescent image
transmission image

[redacted]

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(9) A microfluidic device made of glass would be very hard to seal if an organic membrane is used as one of the components; sealing by fusion bonding would be impossible. Sealing and glueing would be complicated when such a membrane is used even for a device fabricated from a rigid polymeric material.

(10) Staphyloslide™ Latex beads are approximately 0.3 μm in diameter. They are retained by a 0.2 μm membrane, but not a 1 μm membrane.

(11) *Staphylococcus aureas* are approximately 1-μm spheres. We determined permeability of the 1-μm membrane to live bacteria by filtering a suspension of bacteria through the membrane, and using a drop of filtrate to growing colonies of bacteria on an agar plate. We found that the density of colonies obtained from the filtrate was similar (within an order of magnitude) to the density of colonies obtained from the suspension of bacteria that has not been filtered.

(12) Bacteria were grown to a concentration of 10^9/mL and then suspended in PBS buffer for the experiments.

(13) Protein A binds to the Fc region of all IgG antibodies.

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